Hypoxia-driven Hif2a coordinates mouse liver regeneration by coupling parenchymal growth to vascular expansion

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Abstract: Interaction between sinusoidal endothelial cells and hepatocytes is a prerequisite for liver function. Upon tissue loss, both liver cell populations need to be regenerated. Repopulation occurs in a coordinated pattern, first through the regeneration of parenchyme (hepatocytes), which then produces vascular endothelial growth factor (VEGF) to enable the subsequent angiogenic phase. The signals that instruct hepatocytes to induce timely VEGF remain unidentified. Given that liver is highly vascularized, we reasoned that fluctuations in oxygenation after tissue loss may contribute to the coordination between hepatocyte and sinusoidal endothelial cell proliferation. To prevent drops in oxygen after hepatectomy, mice were pretreated with inositol trispyrophosphate (ITPP), an allosteric effector of hemoglobin causing increased O2 release from heme under hypoxic conditions. ITPP treatment delayed liver weight gain after hepatectomy. Comparison with controls revealed the presence of a hypoxic period around the peak of hepatocyte mitosis. Inhibition of hypoxia led to deficient hepatocyte mitosis, suppressed the regenerative Vegf wave, and abrogated the subsequent reconstruction of the sinusoidal network. These ITPP effects were ongoing with the reduction in hepatocellular hypoxia inducible factor 2a (Hif2a). In contrast, Hif1a was unaffected by ITPP. Hif2a knockdown phenocopied all effects of ITPP, including the mitotic deficiencies, Vegf suppression, and angiogenic failure. Conclusions: Oxygen is a key regulator of liver regeneration. Hypoxia—inherent to the expansion of parenchyme—activates Hif2a to couple hepatocyte mitosis with the angiogenic phase. Hif2a acts as a safeguard to initiate sinusoidal reconstruction only upon successful hepatocyte mitosis, thereby enforcing a timely order onto cell type-specific regeneration patterns. These findings portray the hypoxia-driven Hif2a-Vegf axis as a prime node in coordinating sinusoidal endothelial cell-hepatocyte crosstalk during liver regeneration. (Hepatology 2016;64:2198-2209).

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Hypoxia-driven Hif2a coordinates mouse liver regeneration by coupling parenchymal growth to vascular expansion

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Key words: oxygen, hepatocyte mitosis, Vegf, sinusoidal endothelial cell angiogenesis, intercellular crosstalk

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List of Abbreviations:

SEC	sinusoidal endothelial cell
VEGF	vascular endothelial growth factor
ITPP	inositol trispyrophosphate
HIF	hypoxia inducible factor
PHD	prolyl- hydroxylase
ELISA	enzyme-linked immunosorbent assay
qPCR	quantitative real-time polymerase chain reaction
pH3	phospho-histone 3

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Abstract

Interaction between sinusoidal endothelial cells (SECs) and hepatocytes is a prerequisite for liver function. Upon tissue loss, both liver cell populations need to be regenerated. Repopulation occurs in a coordinated pattern, first through the regeneration of parenchyme (hepatocytes) which then produces VEGF to enable the subsequent angiogenic phase. The signals that instruct hepatocytes to induce timely VEGF remain unidentified. Given that liver is highly vascularized, we reasoned that fluctuations in oxygenation after tissue loss may contribute to the coordination between hepatocyte and SEC proliferation. To prevent drops in oxygen after hepatectomy, mice were pretreated with inositol trispyrophosphate (ITPP), an allosteric effector of hemoglobin causing increased O_2-release from heme under hypoxic conditions. ITPP treatment delayed liver weight gain after hepatectomy. Comparison with controls revealed the presence of a hypoxic period around the peak of hepatocyte mitosis. Inhibition of hypoxia led to deficient hepatocyte mitosis, suppressed the regenerative Vegf wave, and abrogated the subsequent reconstruction of the sinusoidal network. These ITPP effects were ongoing with the reduction in hepatocellular Hif2a. In contrast, Hif1a was unaffected by ITPP. Hif2a knockdown phenocopied all effects of ITPP, including the mitotic deficiencies, Vegf suppression and angiogenic failure. **Conclusions:** Our study identifies oxygen as a key regulator of liver regeneration. Hypoxia - inherent to the expansion of parenchyme - activates Hif2a to couple hepatocyte mitosis with the angiogenic phase. Hif2a acts as a safeguard to initiate sinusoidal reconstruction only upon successful hepatocyte mitosis, thereby enforcing a timely order onto cell-type specific regeneration patterns. These findings portray the hypoxia-driven Hif2a-Vegf axis as a prime node in coordinating SEC-hepatocyte crosstalk during liver regeneration.

The reconstitution of liver following tissue loss requires the coordinate regeneration of all liver resident cells.[1] Hepatocytes form the parenchyme, the largest component of the liver, while sinusoidal endothelial cells (SECs) represent the major population of non-parenchymal liver cells. Removal of two thirds of the liver in rodents results in a complete recovery of hepatic volume within about a week[2, 3] and encompasses two principal waves of proliferation.[4, 5] In mouse, hepatocyte mitosis peaks around 48h and is followed by an angiogenic wave that leads to the reconstitution of the sinusoidal network within day 3 and 8 after resection.[1, 6] The conserved sequence of these events points to the existence of a tightly controlled machinery that coordinates the timing of hepatocyte and SEC proliferation. Recent evidence has highlighted the importance of SECs in inducing and regulating parenchymal regeneration.[6, 7] On the other hand, regenerating hepatocytes are the main providers of the key angiogenic molecule VEGF, which is essential for vascular reconstitution after tissue loss.[4-6] What instructs hepatocytes to produce timely VEGF in regenerating liver is unknown.

Hypoxia-inducible factors (HIFs) are prime transcriptional inducers of VEGF, however whether they do so during liver regeneration is unresearched. Under normoxia, the prolyl-hydroxylases (PHD) oxidize HIFs to promote their degradation, while low pO_2 inhibits their hydroxylase activity leading to HIF stabilization and activation. HIFs however can also be activated by other cellular stresses and molecules in an O_2-independent way.[8] In mouse, Hif1a deletion from hepatocytes causes an initial delay in liver regeneration,[9] while global knockout of Phd1 in mice accelerates the hepatocyte cell cycle,[10] implying Hif1a may be a promoter of hepatocellular proliferation.

It has been suggested that the liver remnant following tissue loss is floated with a disproportional volume of portal blood low in oxygen. Because the arterial supply does not change in relative terms,[1] the portal inflow might cause hypoxia, perhaps leading to HIF stabilization. While hypoxia-driven initiation of hepatocyte proliferation is a conceivable view, the contribution of oxygen to the regulation of liver regeneration remains enigmatic.[1], [11, 12]

Here, we sought to clarify the role of hypoxia and HIFs in liver regeneration. More specifically, we explored whether the prevention of a hypoxic state impacts on the recovery of liver following tissue loss, and how hypoxia-dependent alterations in the regenerative process relate to hepatocyte proliferation and the subsequent angiogenic phase.

Materials and Methods

Animals. All animal experiments were in accord with Swiss Federal Animal Regulations and approved by the Veterinary Office of Zürich (KEK-ZH -Nr. 2012-231). Male wild type mice (C57BL6, Harlan, Indiana, USA) aged 10-12 weeks were used and kept on a 12-hour day/night cycle with free access to food and water.

Animal surgery. Isoflurane anesthesia, buprenorphine analgesia, followed by laparatomy and standard partial 68% hepatectomy were performed as described.[2] Following euthanasia, liver/body weight was recorded and organs were preserved in 4% formalin for histopathology or snap frozen in liquid nitrogen. Blood was immediately centrifuged at 5.000 rpm for 6 minutes. Serum supernatant was stored at -80°C until analysis.

Animal treatment. ITPP was synthesized as previously described[13] and i.v. injected 30 min. prior to hepatectomy at 500 mg/kg as a solution (100 µl total volume) containing calcium chloride to balance its chelating effects.[14] Controls received 100 µl 0.9% saline. α*Hif2a*-siRNA and control siRNA were designed & validated by Axolabs Gmbh (Kulmbach, D) and packed into company-owned formulations preferentially targeting murine hepatocytes. We have confirmed preferential targeting in regenerating hepatocytes.[3] *Aha1* was chosen as an internal control knockdown, because it does not yield any phenotype *in vivo* (Axolabs, pers. comm.) and has no apparent impact on liver regeneration.[3] Formulations were i.v.injected 48 hours before hepatectomy. Controls received PBS. Serum liver injury markers were assessed following all treatments to ascertain the lack of significant toxicity.

Enzyme-linked immunosorbent assays. ELISAs for Vegf were from R&D Systems (Minneapolis, USA, DY493, and for Hif1a/Hif2a from NewLife BioChemEX (Bethesda, MD, E90798Mu/E93466Mu). Triplicate measurements were performed. NE-PER Nuclear and Cytoplasmatic Extraction reagents (Thermo Fisher Scientific, Waltham, MA, PK207589) were used to isolate respective subcellular fractions. Separation of cytosolic and nuclear fractions was ascertained through immunoblots[2] for lamin (Santa Cruz Biotechnology, Dallas, TX, sc-377001) and β-tubulin (Cell Signaling, Allschwil, CH, 2128S).

Immunohistochemistry. Antigens were retrieved by citrate boiling of 3-µm archived liver sections. Stainings were performed following standard protocols. Secondary detection was done using the Ventana Discovery

automated staining system and the iView DAB kit (Ventana Medical Systems, Tucson, AZ). Antibodies used were: Hif2a (Abcam, Cambridge, MA, ab199), Hif1a (LifeSpan BioSciences, Seattle, WA, LS-B495), Ki67 (Cell Marque Lifescreen Ltd., Rocklin, CA, CMC27531021), pH3 (Abcam, Cambridge, MA, ab92628) and VE-cadherin (Abcam, ab33168). Pimonidazole stainings (Hypoxyprobe Plus Kit, Hypoxyprobe™, NPI Inc., MA/USA) were done by Sophistolab AG (Muttenz, CH) according to provided instructions. Cell P Analysis software (Olympus, Volketswil, CH) was used for the quantification (per high power field) of positive nuclei and of pimonidazole staining in histological sections. Microvessel density was calculated through the area of VE-cadherin-positive sinusoids (ImageJ software), as this approach has been found an accurate index of vascularization .[15]

Quantitative Real-Time Polymerase Chain Reaction (qPCR). Total RNA was extracted from 50 mg of tissue using Trizol reagent (Invitrogen, Basel, Switzerland). qPCR was performed on cDNA (Thermo Script reverse transcription PCR System, Invitrogen) using an ABI Prism 7500 Sequence Detector system (PE Applied Biosystems Rotkreuz, Switzerland). TaqMan gene expression assays for *Vegfa* (Mm01281449_m1), *Foxm1* (Mm00514924_m1), *Ccna2* (Mm00438063_m1), *Ccnb2* (Mm00438063_m1), *Cdkn1a* (Mm00432448_m1), *Angpt2* (Mm00545822_m1), *Tgfb1* (Mm 01227699_m1), endothelial control *Ld1* (Mm 00775963_m1), and the normalization control 18S *rRNA* (TaqMan control reagents) were from PE Applied Biosystems (Massachusetts, USA). Results represent mean fold induction ($2^{-\Delta Ct}$) ±SD.

Statistical analysis. Data are presented as mean with error bars referring to ±SD. Differences between groups were assessed by a two-tailed t-test assuming unequal variance. Six mice/group were analyzed unless otherwise stated. Differences were considered significant at $P<0.05$. Analyzes were performed using Prism 6.0 (GraphPad).

Results

Treatment with antihypoxic inositol trispyrophosphate delays liver regeneration

To prevent a development of hepatic hypoxia after resection, we applied the novel antihypoxic molecule inositol trispyrophosphate (ITPP). ITPP acts as an allosteric effector of hemoglobin, increasing dissociation of O_2 from heme under low oxygen tension.[13] ITPP efficiently promotes *in vivo* oxygenation of hypoxic tissues, an effect that lasts for about two days following injection.[16] Furthermore, it suppresses Hifs along with the hypoxic response, and displays discernable toxicity neither in animals[14, 17-19] nor humans (http://droia.be/droiacs.html).

We injected ITPP 30 min. before hepatectomy, a period sufficient to inhibit tumor hypoxia without affecting normoxic tissues.[18] To assess regeneration, liver-to-body-weight ratio (LW/BW) was measured at various times after resection. Liver weight regain was significantly delayed between 48h and 96h post hepatectomy in ITPP-treated animals but was comparable to saline controls towards the end of the regenerative process (Fig. 1A). Counting of hepatocytes in cycle (Ki67-positive) revealed no differences between ITPP and control animals (Fig. 1B), suggesting entry into cell cycle is not affected. However, bold nuclear pH3-positivity, marking mitotic hepatocytes,[2] was reduced by ITPP at 48h (the M-phase peak in regenerating mouse liver) and was elevated at later times, consistent with the delayed liver weight gain and corroborated by mitotic counts on histology (Fig. 1C/D). Therefore, ITPP treatment prior to hepatectomy causes a regenerative delay associated with deficient progression of hepatocytes through mitosis.

Marked hypoxia develops with initial parenchymal expansion and correlates with Hif activity

The antihypoxic properties of ITPP should reveal the presence of hypoxia in regenerating liver. The above experiments were repeated with mice pretreated with pimonidazole prior to sacrifice. To detect hypoxia, pimonidazle staining was compared between saline- and ITPP-treated mice at 2h to 96h after resection. For most times, including 2h, pimonidazole positivity was similarly weak. Significant differences were however noted at 32h and 48h, with saline controls displaying marked hypoxic positivity, while hypoxia was suppressed in ITPP-treated mice (Fig. 2A). The hypoxic window hence coincides with the parenchymal S- and M-phase peaks.[2] Next, we measured Hif1a and Hif2a expression. Their mRNA levels did not significantly change during the regenerative course (data not shown). When assessing hepatic protein levels by ELISA, Hif1a was elevated

during the first 48h post resection, however only in the cytosolic but not the nuclear fraction. Moreover, ITPP did not impact on Hif1a levels (Fig. 2B). In contrast, both cytosolic and nuclear Hif2a expression peaked at 32h and 48h post hepatectomy, with ITPP treatment significantly reducing Hif2a levels around these times (Fig. 2C, Supplementary Fig. 1). Therefore, Hif1a transcriptional activity is not promoted by hypoxia in regenerating liver, whereas Hif2a is stabilized and accumulates in hepatocyte nuclei during the hypoxic phase following tissue loss. Likewise, mRNA and protein expression of the Hif target Vegfa was elevated in saline controls but not ITPP-treated mice at 32h/48h (Fig. 2D), suggsting that Hif2a, but not Hif1a, responds to regenerative hypoxia to induce Vegf production. Since Vegf is needed for the subsequent angiogenic phase in regenerating liver,[4-6] we examined sinusoidal vasculature at days 2 to 5 after resection. At 48h, the sinusoidal network was poorly developed and no overt differences were noted between saline controls and ITPP mice. At days 3 to 5 however, sinusoids were prominent in saline controls, but remained marginal in ITPP mice (Fig. 2E). Altogether, these data reveal a consistent correlation between the presence of hypoxia, the activation of Hif2a, the promotion of hepatocyte mitosis, the production of Vegf, and the subsequent reconstitution of the sinusoidal network in regenerating liver.

Hif2a deficiency mirrors the regenerative delay seen following hypoxic inhibition via ITPP

The hypoxia-dependent upregulation of Hif2a and associated changes imply critical function for the molecule in regeneration. In liver, regenerative pathways often act in redundant ways to ensure completion of regeneration also under suboptimal conditions.[20] To perform a loss of Hif2a function experiment, we chose an acute knockdown approach rather than genetic deletion, as to minimize compensatory mechanisms.[20] Additionally, silencing may better mimic ITPP effects, because it does not suppress expression completely. We transfected mice with siRNA using a delivery formulation that in our hands preferrentially targets hepatocytes.[3]

Mice were injected with siRNA against *Hif2a* or the internal control *Aha1* prior to hepatectomy and analyzed. *αAha1*-siRNA did not reduce Hif2a levels after resection. In contrast, *αHif2a*-siRNA efficiently inhibited the cytosolic and nuclear Hif2a peaks in regenerating liver (Fig. 3A, Supplementary Fig. 1). During these times, *αHif2a*-siRNA had no impact on Hif1a expression relative to *Aha1*-controls (Fig. 3B), although transfection appeared to elevate cytosolic levels relative to ITPP/saline treatment (Fig. 2B).

When assessing LW/BW, *Hif2a* knockdown markedly delayed liver weight regain after resection (Fig. 3B). Compared to ITPP (Fig. 1B), the delay in regeneration was extended for about a day, likely due to the more pronounced nuclear Hif2a downregulation through *αHif2a*-siRNA than ITPP around the hypoxic peak (Fig. 3B/2C). Otherwise, the regenerative defect was qualitatively similar; both *Hif2a* knockdown and ITPP caused delayed liver weight regain apparent between 48h and 96h post hepatectomy, implying Hif2a is a crucial mediator of the impact hypoxia has on liver regeneration.

Hif2a promotes hepatocyte mitosis in regenerating liver

Next we assessed whether *Hif2a* knockdown prior to hepatectomy affects proliferative parameters similar to those seen after ITPP treatment. Following *Hif2a* relative to *Aha1* knockdown, no significant differences were noted for Ki67 counts (Fig. 4A). Akin to ITPP-treated mice, bold nuclear pH3 positivity and mitoses were reduced around the mitotic peak through *Hif2a* knockdown. However, the re-elevation of pH3/mitotic counts was delayed after knockdown compared to ITPP treatment (Fig. 1C/D), reflecting the prolonged delay in LW/BW regain following *Hif2a* silencing (Fig. 3C/1A). To confirm a defect in mitotic progression, we measured expression of *Foxm1*, an essential promoter of the hepatocyte S- and particularly M-phase after resection.[21] ITPP treatment suppressed the mitosis-associated peak of *Foxm1* along with its downstream targets *Ccdna2* (cyclin A) and *Ccdnb2* (cyclin B), while upregulating its negative target *Cdkna1* (the cell cycle inhibitor p21) (Fig. 4B).[21] Similar expression alterations were observed upon *Hif2a* knockdown relative to *Aha1* controls. Therefore, Hif2a deficiency phenocopies the mitotic defects associated with ITPP treatment. Together with the similar delay in liver weight gain after hepatectomy and the inhibition of Hif2a nuclear accumulation through ITPP, we conclude that Hif2a activation mediates the mitotic effects hypoxia has on hepatocytes.

Hif2a upregulates hepatocellular Vegf production to induce the angiogenic phase in regenerating liver

Hif2a activity during the hypoxic phase in regenerating liver is likely responsible for the elevations in Vegf observed during this time window. We measured hepatic mRNA and serum protein levels of Vegf following *Hif2a* knockdown and hepatectomy. *αAha1*-siRNA-treated controls displayed both Vegf mRNA and protein increases around the mitotic peak (Fig. 5A), akin to saline-treated mice (Fig. 2D). In contrast, both mRNA and protein elevations were suppressed by *Hif2a* knockdown (Fig. 5A), again mirroring the effects seen with ITPP treatment (Fig. 2D). When examinig vasculature at 48h to 120h after hepatectomy, reconstitution of the

sinusoidal network was apparent in Aha1 controls, but was defective in the *αHif2a*-siRNA-treated animals (Fig. 5B). To substantiate a functional deficiency in vessel formation, we measured microvessel density (MVD) after *Hif2a* knockdown. Knockdown suppressed the raises in MVD seen in controls after 48h, similar to ITPP (Fig. 5C). Furthermore, to assess angiocrine function, we measured the expression of endothelial *Angpt2*, important for sinusoidal proliferation,[7] and its endothelial downstream target *Tgfb1*, a key angiocrine signal required for the termination of parenchymal regeneration.[7] At 48h after resection, *Hif2a* knockdown - akin to ITPP - had no impact on Angpt2 and Tgfb1. In contrast, both *Hif2a* knockdown and ITPP impaired the upregulation of *Angpt2* and *Tgfb1* seen in controls at 120h following hepatectomy. Altogether, these findings indicate that Hif2a activity is required to induce Vegf production and the subsequent angiogenic phase for the regeneration of SECs.

Discussion

Intense exchange between hepatocytes and circulating blood is key to liver function. Exchange is enabled through the specific liver architecture, where each hepatocyte lines an endothelial cell of the sinusoidal network. The intimate bonds between hepatocytes and SECs, combined with the profound changes in hepatic blood flow following tissue loss, have fostered the view that declines in oxygen levels may influence the regenerative process. Thus far, direct evidence for a role of hypoxia in liver regeneration was lacking. In this study, we show that after hepatectomy, (i) significant hypoxia develops with parenchymal growth and is required for efficient liver recovery; (ii) the hypoxic phase leads to hepatocellular Hif2a, but not Hif1a, activation; (iii) Hif2a promotes the progression of hepatocytes through mitosis; and (iv) Hif2a in parallel induces the major wave in Vegf needed for the subsequent reconstitution of the sinusoidal network.

To explore the role for oxygen in the regulation of liver regeneration, we took advantage of the nontoxic antihypoxic molecule ITPP. Acting on hemoglobin, ITPP promotes the oxygenation of hypoxic tissue without apparent effects on well-oxygenated regions.[13, 18] Consequent to its dependence on hemoglobin, ITPP seems to lack direct effects on cells. Testing ITPP on epithelial cell cultures, we have not observed any effects on proliferation, cell death, mitochondrial/glucose metabolism, and migration.[16] Therefore, ITPP represents a unique tool to study the impact a hypoxic environment has on biological processes *in vivo*.

ITPP treatment delayed liver regeneration during a period which we have previously shown to be associated with deficient hepatocyte mitosis.[2] Notably, early time points were not affected (i.e. weight gain, Ki67), suggesting that immediate hypoxia - as postulated based on portal flow changes[1] - plays a minor, if any, role in the regulation of liver regeneration. We detected only small difference in pimonidazole staining at 2h after resection, implying immediate hypoxia may be mild. Instead, distinct hypoxia was detectable between 24h and 48h, when sinusoidal density is known to be lowest (see also Fig. 5C & Supplementary Fig. 2) and hepatocyte proliferation is peaking.[6, 20, 22] Conceivably, the selective expansion of parenchyme is the cause of tissue hypoxia, akin to what is known from tumors. In turn, parenchymal hypoxia activates Hif2a, which, as shown by the comparison with ITPP and knockdown, promotes the progression of hepatocytes through mitosis.

Notably, ITPP is considered for the treatment of malignancy,[14-17] and we have initiated the first clinical trial of ITPP in cancer patients (NCT02528526). In experimental colorectal liver metastasis, we have observed a marked

inhibitory effect of ITPP and demonstrated its ability to potentiate standard chemotherapy.[16] ITPP hence might be used for the downstaging of liver tumors to enable resection. Here, our current findings suggest that ITPP treatment would need cessation before resection, as otherwise patients may be put at risk of resection-induce liver failure. Indeed, when performing an extended (86%) hepatectomy inducing mild liver failure,[2] prior ITPP treatment increases mortality from 20% to 100% (Supplementary Fig. 3), demonstrating the associated risk of postoperative liver failure. On the other hand, these findings also demonstrate the potent proregenerative function[2] hypoxia has after tissue loss.

As a limitation of our study, only one siRNA was used for knockdown; we hence cannot exclude off target effects. However, the strongly homologous Hif1a[8] was unaffected by Hif2a knockdown. Furthermore, Hif2a suppression by either knockdown or ITPP treatment had very similar outcomes across all investigated parameters related to liver weight gain, cell cycle progression and endothelial regeneration. Therefore, the sum of evidence implies that the main phenotype observed after knockdown is dependent on Hif2a. Finally, siRNA effects on extrahepatic tissues and non-parenchymal liver cells are likely minimal, because our transfectant preferentially targets hepatocytes,[3] and because nuclear Hif2a expression appeared to be low and unaffected by knockdown in both, other tissues (Supplementary Fig. 1) and non-parenchymal cells (Supplementary Fig. 4).

Our observation that Hif2a, but not Hif1a, mediates hypoxia-driven proliferation in regenerating liver is not unexpected. Diseases typified by HIF overactivity due to mutation in the HIF-degrading enzyme VHL present with hepatomegaly and hypervascularity, both of which have been attributed to HIF2A but not HIF1A.[23, 24] Moreover, during early liver organogenesis, zebrafish Hif2a, but not Hif1a, promotes hepatocyte proliferation required for the provision of the future parenchyme mass,[25] suggesting the mitotic function of HIF2A is conserved in liver. Puzzlingly, Hif1a knockout does delay liver regeneration in mice. However, Hif1a also regulates glucose metabolism after resection, with delayed regeneration perhaps being an indirect effect related to a perturbed energy metabolism.[9] Furthermore, we measured very low Hif1a nuclear levels that were unresponsive towards ITPP-induced oxygenation changes. The reasons for the low hepatic Hif1a expression are unclear, however Hif1a levels may be suppressed by *miR122*, the most abundant microRNA in liver.[26] Consistent with its marginal nuclear expression, Hif1a - unlike Hif2a - does not react to hypoxia-reoxygenation in hepatocyte cultures.[27] We hence conclude that Hif2a, but not Hif1a, is a main mediator of the hypoxic response in regenerating liver.

An intriguing finding of this study is the dual fuction of Hif2a. Its genetic or antihypoxic inhibition not only suppressed the peak of hepatocyte mitosis, but also abrogated the dominant wave of Vegf elevations in regenerating liver. As shown in previous studies,[4, 5, 27] the Vegf wave is essential for sinusoidal regeneration and is sourced from replicating hepatocytes. Therefore, hypoxia developing from parenchymal regeneration functions in analogy to tumor-associated hypoxia, inducing Vegf as to re-install blood supply. Hif2a in turn provides a simple mechanism to combine Vegf production with hepatocellular proliferation. This parallel regulation of hepatocyte mitosis and the subsequent angiogenic phase identifies Hif2a as a central node in coordinating the regeneration of different liver cell populations. By coupling hepatocyte mitosis to Vegf production, Hif2a appears to act as a safeguard to permit the course of the angiogenic phase only upon successful parenchymal growth. Regenerative hypoxia and Hif2a are hence necessary to imprint a timely order onto cell-type specific regeneration patterns. This timing of hepatocyte and SEC proliferation however can be overruled, since after *Hif2a* knockdown, liver eventually grows back to its original size, revealing the presence of compensatory mechanisms that are securing vital liver function. Interestingly, *Hif2a* knockdown and ITPP suppressed the upregulation of endothelial *Tgfb1*, active late after hepatectomy to terminate hepatocyte proliferation.[1, 7] It is hence tempting to speculate that failed *Tgfb1* upregulation is a crucial event enabling the late-phase compensatory parenchymal growth seen after *Hif2a* knockdown or ITPP treatment.

The interplay between hepatocytes and SECs though Hif2a integrates into the previously described relationships between parenchymal and endothelial regeneration. Angiocrine signals from SECs are essential for the induction, progression and completion of liver regeneration. Via early Vegf signaling, SECs provide Hgf and Wnt2 to induce parenchymal regeneration.[6, 28] Moreover, Vegf stimulates parenchymal proliferation also via hepatocellular Vegfr1 engagement.[28] Through downregulation of Angpt2, SECs further hinder their own proliferation and lower production of Tgfb1, the key inhibitor of hepatocyte proliferation.[7] Following the major phase of parenchymal growth, intensified Vegf signaling is needed for SEC proliferation.[6] The concomitant re-elevations in Angpt2 facilitate sinusoidal regeneration and upregulate Tgfb1 to cease hepatocyte proliferation.[7] Thus, two separate angiocrine phases exist that are characterized by divergent signaling to hepatocytes. Via Hif2a, the growing hepatocyte mass produces the Vegf amounts needed for SEC proliferation, thereby providing a link between the two distinct angiocrine phases. The hypoxia-driven Hif2a signaling illustrates the conceptual simplicity behind the timely coordination of hepatocyte-SEC regeneration after tissue loss, perhaps reflecting the evolutionary need to first replicate the functional units of liver, the hepatocytes, and then the sinusoids as a simple consequence of the developing tissue hypoxia. In summary, our findings portray the hypoxia-induced

Hif2a-Vegf axis as a prime regulator of the regenerative SEC-hepatocyte crosstalk and reveal a crucial role for oxygen during liver regeneration.

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Figure legends

Figure 1. Treatment with ITPP delays liver regeneration after hepatectomy. (A) Liver-to-body weight ratio (LW/BW) in saline- and ITPP-treated mice after resection. Counts of (B) hepatocytes with nuclear Ki67-positivity, of (C) hepatocytes with bold nuclear pH3-positivity, and of (D) mitotic hepatocytes on histology in liver of saline/ITPP-treated mice. Note the re-elevation in pH3/mitotic counts at later time points, consistent with the accelerated liver weight regain at 120h post resection in ITPP-treated mice. N=6 mice/group. *P<0.05, **P<0.005.

Figure 2. ITPP treatment inhibits hypoxia, Hif2a activation, Vegf production and sinusoidal reconstitution after hepatectomy. (A) Quantification of pimonidazole staining in liver from saline/ITPP-treated mice between 2h and 96h after hepatectomy, with representative stainings shown below. (B) Hif1a and (C) Hif2a cytosolic (*top*) and nuclear (*bottom*) protein levels in liver from saline/ITPP-treated mice after resection. (D) Vegf hepatic mRNA and serum protein levels in saline/ITPP-treated mice after resection. (E) VE-cadherin staining of the sinusoidal network between 48h to 120h after resection. Note the paucity of vessels in both saline (cont) and ITPP-treated liver at 48h post resection. For better visualization, image colors unrelated to brown were desaturized. N=6 mice/group. *P<0.05, **P<0.005.

Figure 3. Hif2a knockdown and its impact on liver weight gain after hepatectomy. (A) Cytosolic (*top*) and nuclear (*bottom*) Hif2a levels in liver from *αAha1*-siRNA (Aha1) or *αHif2a*-siRNA-treated mice after hepatectomy. (B) Cytosolic (*top*) and nuclear (*bottom*) Hif1a levels in liver after *Hif2a*/*Aha1* knockdown and resection. (C) Liver weight gain (LW/BW) following *Hif2a*/*Aha1* knockdown and hepatectomy. Note the prolonged delay in weight regain after *Hif2a* knockdown relative to ITPP treatment (Fig. 1C), consistent with the stronger Hif2a suppression through knockdown than ITPP (Fig. 2C). *Aha1* knockdown somewhat prolonged nuclear Hif2 elevation, however without effect on LW/BW. N=6 mice/group. *P<0.05, **P<0.005.

Figure 4. Hif2a deficiency inhibits progression through mitosis in regenerating liver. (A) Counts of hepatocytes positive for Ki67, pH3, or undergoing mitosis in liver of mice following *Hif2a*/*Aha1* knockdown and hepatectomy. N=6 mice/group. (B) Relative expression of genes encoding Foxm1, cyclin A, cyclin B, and p21 during the first 4 days after hepatectomy. Expression is shown for ITPP/saline-treated mice (*left graphs*) and for *αHif2a*-siRNA/*αAha1*-siRNA-treated mice (*right graphs*). N≥3 mice/group. *P<0.05, **P<0.005.

Figure 5. Hif2a induces Vegf production for sinusoidal reconstitution in regenerating liver. (A) Hepatic Vegf mRNA and serum protein levels following *Hif2a*/*Aha1* knockdown and hepatectomy. (B) VE-cadherin staining of sinusoidal vessels between 48h to 120h after knockdown and resection. (C) Microvessel density after *Hif2a*/*Aha1* knockdown and hepatectomy in comparison to ITPP and saline treatment. (D) Relative expression of genes encoding endothelial *Angpt2* and *Tgfb1* after hepatectomy. ITPP/saline treatment is shown to the left, *Hif2a*/*Aha1* knockdown to the right. Expression values were normalized to a generic control (upper panels) or to an endothelial control (*Id1*, see ref. 7). The similar expression patterns are consistent with the reported expression of *Angpt2*-*Tgfb1* on endothelial cells. N=6 mice/group. *P<0.05, **P<0.005.